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HORMONAL CONTROL OF REPRODUCTION IN *BUSYCON*: II. LAYING OF EGG-CONTAINING CAPSULES CAUSED BY NERVOUS SYSTEM EXTRACTS AND FURTHER CHARACTERIZATION OF THE SUBSTANCE CAUSING EGG CAPSULE LAYING

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Abstract

Ram (1977) previously observed that *Busycon* would lay empty egg capsules when injected with nervous system extracts and suggested that egg capsules containing eggs would be laid only after a number of eggless capsules had first been laid. Data supporting this suggestion and characterizing the gel filtration behavior and species specificity of the substance causing capsule laying are reported.

Busycon capsule strings collected in the field always had empty egg capsules at the initially laid end (*B. carica*, 13–17; *B. canaliculatum*, 4–57). *B. carica*, collected while laying in the field, continued to lay capsules in the laboratory at the average interval of 1.9 ± 1.5 hours/capsule.

Injection of nervous system extracts into *B. canaliculatum* caused capsule laying. The least amount that would cause capsule laying was $^1/_{16}$ of a nervous system. Injection of ¼ nervous system every two or three hours resulted in the laying of egg-containing capsules after a series of four or more empty capsules. The number of initial empty capsules was correlated with the percent of injections causing capsule laying. The percentage of hard capsules increased from 36 ± 24% before eggs were inserted to 59 ± 27% after eggs were inserted ($P < 0.05$).

The substance causing capsule laying in *Busycon* eluted from Sephadex G-50 at the same position as *Aplysia* egg laying hormone; however, cross-injection experiments between the two species failed to cause egg or capsule laying. Nervous system extracts from *Strombus gigas* caused capsule laying in *Busycon*; whereas, *Busycon* nervous system extracts did not cause laying in *Strombus*.

Introduction

Large prosobranch gastropods, including *Busycon, Strombus,* and *Haliotis*, are commercially important sources of protein and ornamental shells. Efficient exploitation of these animals, and possibly development of mariculture for them, may depend on increased knowledge of mechanisms controlling their reproduction, the knowledge of which might be exploited to produce spawn at desired intervals, in increased numbers, and/or beyond the natural spawning seasons. For *Haliotis*, spawning can be reliably triggered by exposure of ripe females to hydrogen peroxide in alkaline sea water (Morse, *et al.*, 1977), a method that did not work in *Strombus* (Morse, *et al.*, 1978). For *Busycon*, Ram (1977) reported that injection of nervous

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Abbreviation: central nervous system without the visceral ganglion, dissected along with a short piece of esophagus, CNS.

system extracts caused the laying of egg capsules, which were, however, devoid of eggs. In the present report, a method is demonstrated for obtaining egg-containing capsules in *Busycon*. In addition, the substance that induces the laying of egg capsules by *Busycon* is further characterized, and the species specificity of nervous systems inducing the response is examined.

In the field, *Busycon* ordinarily lays long strings of egg capsules over periods of several days (Magalhaes, 1948). This contrasts with the laying of egg capsules caused by nervous system extracts, in which usually only a single eggless capsule is laid in response to the injection of an extract of one quarter of a nervous system. Ram (1977) proposed that insertion of eggs into egg capsules might occur only when the substance(s) causing egg capsule laying had been working over longer periods than were used in his experiments. According to this proposal, an animal would be expected to lay a number of eggless capsules (perhaps 5 to 15), on the first day of capsule laying, before eggs would be inserted in subsequent capsules. Two capsule strings laid spontaneously in the laboratory seemed to confirm this proposal, since the initial 8–11 capsules laid during the first day of laying contained no eggs, after which eggs appeared in increasing numbers in subsequent capsules.

Although empty capsules in *Busycon* egg strings had been noted by Magalhaes (1948), she gave no indication of the location of the empty capsules in the string. The location of the empty capsules is critical to this proposal; there must be several at the beginning of the string. Therefore, the first data presented concerning this proposal are on the distribution of the number of eggs in egg capsule strings collected in the field. A more direct test of the proposal is to inject animals with active nervous system extracts a greater number of times per day than in previous experiments to see if eventually, after laying a number of empty capsules, the animals would begin laying capsules with eggs. In the experiments of Ram (1977) the maximum frequency and number of injections in a day was two injections spaced three hours apart, and no eggs were obtained. When the number of injections per day was increased to eight to twelve, as described in this paper, capsules containing eggs were laid.

Materials and Methods

Capsule strings of both *Busycon canaliculatum* and *Busycon carica* were collected in Nantucket Sound in July, 1978 and August, 1981. The collection area was near Cotuit Bay in a relatively homogeneous, current-swept sandy region in depths ranging from 1–4 meters. This area is about 22 km east of Woods Hole on the south side of Cape Cod, Massachusetts.

The end of the capsule string attached to the substrate was noted and the number of eggs per capsule was counted at regular intervals along the string. Observations in the field of animals still in the process of laying capsule strings clearly showed that the end of the capsule string attached to the substrate was laid first. Several *B. carica* still in the process of laying eggs were brought back to the laboratory to observe the capsule-laying rate.

Experiments were done at the Marine Biological Laboratory, Woods Hole, Massachusetts during July and August, 1981. Specimens of *Busycon canaliculatum*, collected locally by the Marine Biological Laboratory Marine Resources Department, were maintained in running sea water at the ambient temperature of 20–25°C.

Nervous systems for extracts were obtained from animals having a shell length greater than 13 cm, without regard to sex, since previous studies (Ram, 1977) had

shown that nervous system extracts from both sexes could induce capsule laying. For all experiments, extracts were made from the central nervous system without the visceral ganglion, dissected along with a short piece of esophagus as described previously (Ram, 1977), and designated CNS. Dissected CNSs were immediately placed on ice and frozen at −15°C until used in making an extract. Eight to twenty-four CNSs were thawed, homogenized in a motor-driven glass-teflon homogenizer on ice with 0.1 to 0.3 ml filtered sea water per CNS, and then placed on a boiling water bath for 10 min. After cooling, the boiled homogenate was centrifuged 25 min at 10,000 × *g*, the supernatant was diluted with sea water to obtain 0.3 or 0.4 ml/one quarter CNS, and then this CNS solution was pipetted into several test tubes (usually 3 or 4 times the volume of one quarter CNS in each test tube), which were then stored at −15°C until needed for injection into recipient animals. Animals were injected through the side of foot, as described previously (Ram, 1977).

Since large *Busycon* are always female (Ram, 1977), large animals (shell length greater than 17 cm, wet weight without shell = 150–300 g) were used as recipients of nervous system extract injections. Initially, thirty large females were screened to find animals that would lay in response to injection of one quarter CNS. Not all large females lay in response to nervous system extract injection, a finding that is related in some animals to lack of sexual maturity (small gonad index, Ram, 1977); however, animals that respond once are usually responsive to subsequent injections (Ram, 1977). Thus, this screening procedure identified responsive animals to be used as recipients in further experiments.

For gel filtration of nervous system extracts, eight CNSs were homogenized and boiled in 1 ml sea water, as above. The aqueous phase after boiling was centrifuged at 20,000 × *g* for 25 min. Gel filtration of 200 μl of the supernatant was done on Sephadex G-50 equilibrated in sea water in a 0.8 × 29 cm polystyrene column. Fractions were approximately 1.2 ml (one run) and 0.8 ml (a second run). Optical density of all fractions at 280 nm was measured, and all fractions were frozen at −15°C until later bioassay. To bioassay fractions for ability to cause capsule laying the entire fraction was injected into recipients. Elution pattern was compared to the elution of marker proteins, *Aplysia* egg-laying hormone, and blue dextran run on a Sephadex G-50 column of identical dimensions (Ram, 1982a).

The species specificity of the substance causing capsule laying was examined by injecting nervous system extracts of *Aplysia californica* and *Strombus gigas* into *Busycon*, and *vice versa*. *Aplysia californica* (300–500 g) was obtained from Pacific Biomarine Laboratories. The abdominal ganglion of *Aplysia* is known to contain a 4385 dalton polypeptide that causes egg laying upon injection into mature *Aplysia* (Chiu *et al.*, 1979). Sea water homogenates of abdominal ganglia (one ganglion per ml) from *Aplysia* were made, and seven *Busycon* were injected with 0.5 ml each. As described in results, 0.5 ml aliquots of similarly prepared homogenates injected into *Aplysia* showed that sufficient egg-laying hormone to cause egg laying in *Aplysia* was present in such homogenates. Conversely, 0.5 ml aliquots of *Busycon* extracts containing one CNS per 0.5 ml were injected into each of four mature *Aplysia* which were known not to have laid eggs for at least 48 hours before injection. For these experiments *Aplysia* were maintained for three days in running sea water at ambient temperatures and were fed lettuce.

Strombus were obtained from C. Berg (Marine Biological Laboratory) who collected them in the Bahamas in August, 1981. *Strombus* CNS extracts, containing one CNS per 0.5 ml, were prepared in a manner identical to that described for *Busycon* extracts, and 0.5 ml was injected into each of three *Busycon*. Conversely, 0.5 ml of *Busycon* extracts containing in one experiment ¼ CNS per 0.5

ml and in another experiment one CNS per 0.5 ml were injected into each of six *Strombus*.

Results

Spontaneously laid capsules: location of eggs in capsule strings and rate of laying

Capsules at the beginning of egg strings were always devoid of eggs. The distribution of egg number over several entire capsule strings in *B. carica* (n = 5 strings) and *B. canaliculatum* (n = 3) is shown in Figure 1. The median number of empty egg capsules at the beginning of a string was 15 (range 13–17) for *B. carica* and 5 (range 4–57) for *B. canaliculatum*. Additional features of the pattern are (a) the number of eggs reaches a plateau (*i.e.* egg numbers neither increasing nor decreasing more than 5–15 eggs/capsule) by about capsule number 20 or 30, and (b) at the end of the string the number of eggs/capsule either gradually

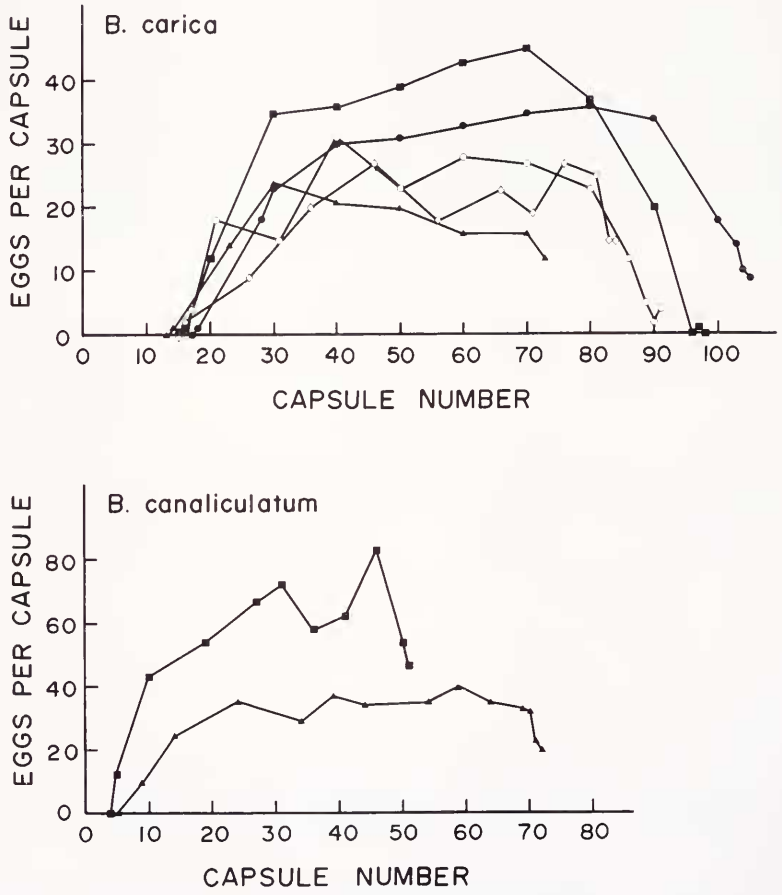

FIGURE 1. Distribution of eggs in *B. carica* and *B. canaliculatum* capsule strings collected in the field. The first point in each curve is for the last capsule containing no eggs; all previous capsules had no eggs. For *B. canaliculatum*, one other capsule string, containing 57 capsules, had no eggs in any capsule.

decreases to near zero over the last 10 to 20 capsules, or alternatively, capsule laying ends abruptly at or near the plateau level of eggs.

Capsule laying rates were observed in six *Busycon carica* brought back to the laboratory. The average interval between laying egg capsules was 1.9 ± 1.5 hours (Table I).

Screening recipients and dose-response analysis

As in the study by Ram (1977), not all large females laid egg capsules in response to injection of nervous system extracts. In the present study, injection of one quarter CNS caused capsule laying in response to the first injection in 9 of 30 recipients (30%). Animals that had been induced to lay once usually laid in response to subsequent nervous system extract injections, although as described below, some were more reliable than others. Experiments described below used only animals chosen from these nine "reliable layers."

To test the possibility of using a dose lower than one quarter CNS, which was the usual amount used by Ram (1977), dose-response analysis was done. CNS extracts prepared by the standard procedure were serially diluted in sea water to produce extracts containing $1/64$, $1/32$, $1/16$, $1/8$, and $1/4$ CNS per 0.5 ml. Six recipients were each injected with 0.5 ml of these solutions, starting with the lowest concentration and increasing the concentration on each subsequent injection, with injections made no more frequently than once every four hours. The number of recipients laying at each dose was as follows: 0, $1/64$; 0, $1/32$; 2, $1/16$; 4, $1/8$; 6, $1/4$. Thus, the threshold dose to induce capsule laying was between $1/16$ and $1/4$ CNS. Consequently, it seemed inadvisable to inject less than $1/4$ CNS in subsequent experiments as lower concentrations would be at or near the point at which capsule laying would fail.

Laying of egg-containing capsules caused by injections

Busycon were injected repeatedly with one quarter CNS every three hours (Experiment I) or every two hours (Experiment II). These rates of injection were

TABLE I

Average interval between laying capsules during spontaneous capsule laying.

Animal number	Number of capsules	Time to lay (h)	Hours/capsule
1	10	16	1.6
2	7	10	1.4
3	39	88	2.3
4	11	4	0.4
5	6	6.5	1.1
6*	34	156	4.6
Mean ± S.D.	18 ± 15	47 ± 62	1.9 ± 1.5

* Number of capsules was also counted for this animal 67 hours after bringing the animal into the laboratory, at which time 19 additional capsules had been laid, *i.e.* an average interval of 3.5 hours/capsule.

B. carica, collected while laying in the field, continued to lay in the laboratory. The number of capsules in the string was counted when animals were first brought into the laboratory, and the total number of capsules was counted again shortly after capsule laying was completed. The table gives the difference of these two numbers. This may have overestimated the number of capsules laid during the period of observation by one to three, since several capsules are normally obscured by the foot during laying.

chosen because they were comparable to the frequency of capsule laying observed in spontaneous capsule laying. As described below, the higher rate of injection was more successful at causing the insertion of eggs into egg capsules.

Experiment I. The four most sensitive recipients from the dose-response experiment were injected with one quarter CNS every three hours for 66 hours. Three of the four animals laid capsules after every injection; the fourth laid capsules after 17 of 22 injections (77%) and in no case missed laying two times in a row. Of the former animals, one began putting eggs in egg capsules at capsule number fourteen, which contained nine eggs. The number of eggs per capsule in subsequent capsules was 0, 13, 6, 7, 30, 0, 8, 9, and not counted. No other animal laid egg-containing capsules.

Experiment II. Six animals were recipients, including two animals from the previous experiment (one which had laid eggs and one which had not). Animals were injected with one quarter CNS every two hours for up to 42 hours. Injections into egg-laying animals were discontinued when three or more egg containing capsules had been laid. Since the latency to induce capsule laying by extract injection is greater than two hours (Ram, 1977), capsules did not appear until after the second injection (presumably the capsule caused by the first injection). After the last injection, two capsules were laid about two hours apart. Taking this delay into account, three animals, including the two from the previous experiment, laid capsules after every injection. Two other recipients laid capsules after 85%, 75%, and 69% of injections, and only the last animal missed more than one capsule in a row.

Five of the six recipients in this experiment began putting eggs in capsules after laying several empty capsules. The number of eggs per capsule for each egg layer is plotted in Figure 2. The median number of initial empty capsules was six (range 4–9). The number of eggs per capsule varied from one up to 82. The animal that

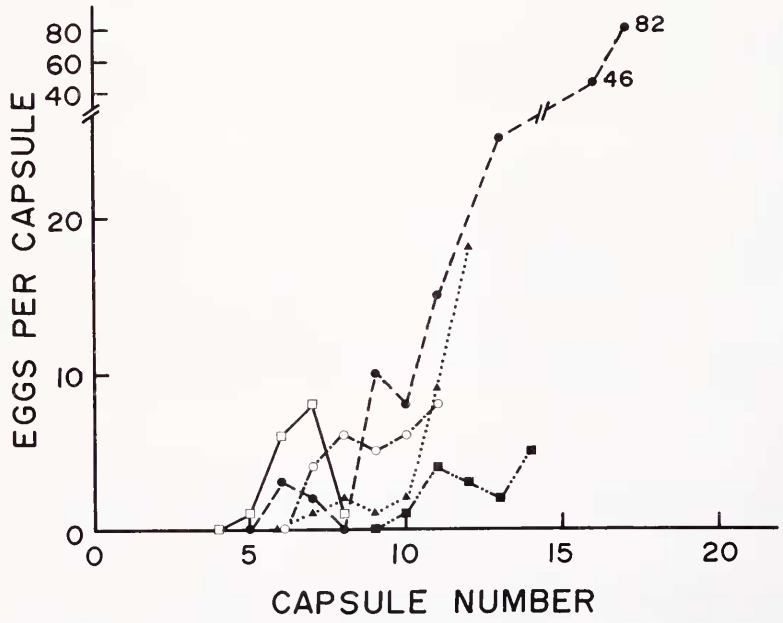

FIGURE 2. Number of eggs per capsule for capsules laid in response to injection of extracts of one quarter CNS every two hours. The first point in each curve is for the last capsule containing no eggs; all previous capsules had no eggs.

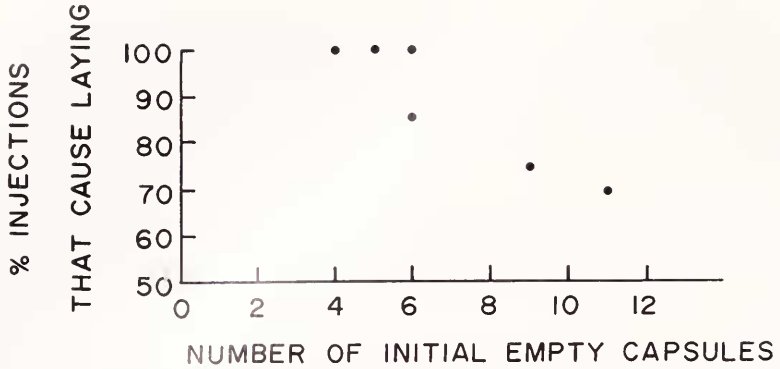

FIGURE 3. Correlation between the percent of injections that caused capsule laying and the number of empty capsules laid prior to laying capsules with eggs, for animals injected with one quarter CNS every two hours. The data illustrated include a point (69%, 11 capsules) for one animal which laid only eggless capsules (eleven) and for which only 16 injections were given. Conceivably, a larger number of empty capsules would have been laid if injections had been continued. Using the data illustrated, $r = -0.93$ and $P = 0.003$. Even if a larger number than 11 eggless capsules is used for the animal that did not lay eggs, the P level is <0.05 if the number of eggless capsules is <40. Without this point, the statistics for the correlation are $r = -0.87$, $P = 0.02$.

did not put eggs into its capsules was the one that laid capsules only 69% of the time; injections into this animal were discontinued after 16 injections. There is a significant correlation ($P < 0.05$) between probability of laying an egg capsule and the number of initial empty capsules (Fig. 3).

Shape of the capsules. As in the study of Ram (1977) animals laid either hard well-formed capsules or soft bulb-like capsules, depending on the success of the animal in passing the capsule into the pedal pore. Eggs were inserted into both hard and soft capsules; however, the proportion of capsules that were hard increased significantly after the animals began inserting eggs. Overall, the percentage of hard capsules increased from 36 ± 24% (mean ± standard deviation) before eggs were inserted to 59 ± 27% after eggs were inserted ($P < 0.05$, Wilcoxon signed ranks test).

The observation that egg-filled capsules were more likely to be hard probably cannot be explained simply by supposing that the animal becomes more likely to make hard capsules with each succeeding capsule. For example, for the animals that eventually inserted eggs in their capsules there was no significant difference in the percent of hard capsules among the first half of the initially laid eggless capsules (33 ± 27%) and the second half of the initially laid eggless capsules (38 ± 22%). Moreover, three animals that laid only eggless capsules did not lay more hard capsules at the end of the experiment than at the beginning.

Gel filtration

Figure 4 shows the optical density pattern of *Busycon* nervous system fractions eluted from a Sephadex G-50 column. Bioassays were done on all fractions eluting from just before the void volume peak to three fractions after the amino acid peak, and egg capsule laying was obtained only from fractions for which $V_e/V_0 \simeq 1.5$ ($n = 2$ column runs). The substance causing capsule laying eluted from the column at approximately the same place as would be expected for *Aplysia* egg-laying hormone.

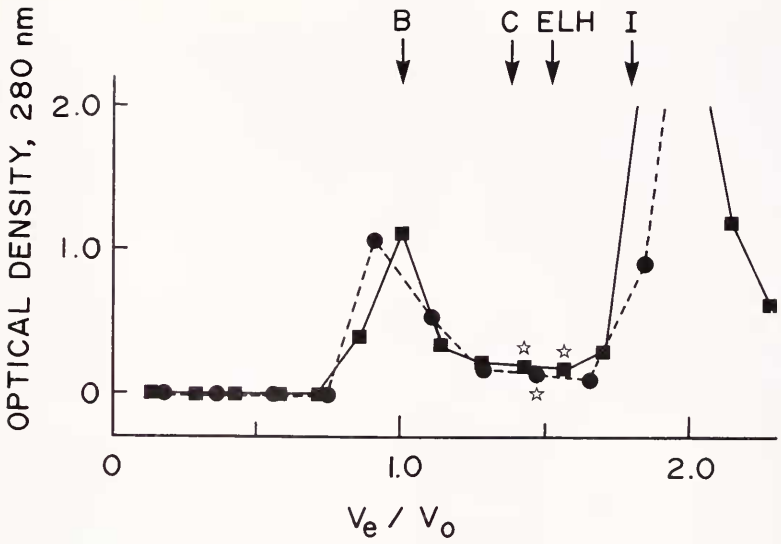

FIGURE 4. Sephadex G-50 gel filtration in sea water of *Busycon* nervous system extracts. The optical density of fractions from two separate runs (● and ■) is shown, and fractions that caused capsule laying upon injection into reliably laying *Busycon* are indicated (☆). For each run, all fractions from just before the void volume peak to three fractions after the amino acid peak were tested in the bioassay. The elution volume for markers run on a Sephadex G-50 column with identical dimensions and eluant (sea water) are indicated at the top: B, blue dextran (void volume); C, cytochrome c, 11,400 daltons; ELH, *Aplysia* egg-laying hormone, 4385 daltons; I, insulin B chain, 3,540 daltons.

Species specificity

Aplysia. Injection of one *Busycon* CNS into each of four *Aplysia* failed to cause egg laying. Three of the four recipients were tested three hours later with *Aplysia* abdominal ganglia extracts (one half ganglion per recipient), and all three began laying within 90 min. Thus, *Busycon* CNS extracts differ significantly from *Aplysia* abdominal ganglia extracts in causing egg laying in *Aplysia* ($P = 0.028$, Fisher exact probability).

Injection of half of an *Aplysia* abdominal ganglion into each of seven *Busycon* failed to cause capsule laying. All seven *Busycon* laid capsules on subsequent injection of *Busycon* CNS extract. Thus, *Aplysia* abdominal ganglia extracts differ significantly from *Busycon* CNS extracts in causing capsule laying in *Busycon* ($P = 0.00029$, Fisher exact probability).

Strombus. Injection of one *Strombus* CNS into each of three *Busycon* caused laying of empty egg capsules by two of the recipients. This result is thought to be highly significant since a false positive result has never occurred in any previous experiment; *i.e.* in all cases where responsive *Busycon* were injected with a solution known to contain little or no nervous system extract, no capsule laying was obtained (*e.g.* sea water and phosphate controls in Ram (1977); low doses in the dose-response analysis and pre-void volume fractions in gel filtration studies in this paper).

In the opposite direction, neither ¼ *Busycon* CNS nor one *Busycon* CNS injected into each of six recipient *Strombus* caused the laying of eggs or egg capsules. Failure to elicit laying in *Strombus* following *Busycon* CNS injection could have been due to insufficient dosage or to a lack of readiness to respond in the recipients.

Discussion

The present study examined the proposal that *Busycon* would insert eggs into egg capsules only after a number of empty egg capsules were laid. The results support this proposal with both field and experimental data, showing (a) in the field, *Busycon* laid four or more empty capsules before egg-containing capsules were laid, and (b) when injected once every two hours with nervous system extracts that cause capsule laying, *Busycon* began laying egg-containing capsules after 4 to 9 empty capsules were laid.

A correlation was found between the number of initial empty capsules and the overall probability of laying egg capsules, and might be explained by supposing that the reproductive state of the animal in some way could influence both. Alternatively, this observation may have the trivial explanation that slow animals simply were not being injected as efficiently as fast ones, a circumstance that would be expected to decrease the number of capsules as well.

The correlation of the probability of hard capsules with the insertion of eggs is not so easily explained. Is there some internal feedback by which the animal senses when eggs have been inserted and therefore tries harder to move the capsule to the pedal pore? Whatever the explanation, it appears to be important for development of the eggs, as eggs in soft capsules degenerated, and such capsules fell apart within two weeks of being laid.

If the procedure developed here is to be of use in bringing *Busycon* reproduction under experimental control, it is necessary that the eggs be viable. Due to limited time, it was not possible to do a thorough study of this; however, it was observed that of the eggs laid in hard capsules in Experiment I, about half the eggs in each capsule had developed to the four cell stage by 10 days after laying. This rate of division is comparable to that seen in spontaneously laid eggs (Jonathan P. Davis, unpublished observations).

When capsules from Experiment II were maintained for 30 days, bacteria apparently invaded most of them; however, several contained normally developed young *Busycon.* This rate of development is comparable to that seen by one of us (Jonathan P. Davis, unpublished observations) in *B. carica* capsule strings freshly laid in the laboratory. Thus, it appears that eggs laid as a result of nervous system extract injections are fertile and capable of developing normally. Further experiments on optimal conditions for achieving this seem appropriate.

Induction of egg laying by injection of nervous system extracts has previously been demonstrated in *Aplysia* (Kupfermann, 1970; Toevs, 1970), *Pleurobranchaea* (Davis, *et al.*, 1974; Ram, *et al.*, 1977), *Lymnaea* (Geraerts and Bohlken, 1976), *Stylocheilus* (Ram, 1982a, b), and *Dolabrifera* (Ram, 1982b). The egg laying of these gastropods in response to nervous system extracts differs from the behavior of *Busycon* in two significant ways: (a) eggs are laid in response to a single injection, and (b) the egg-laying episodes induced by nervous system extracts are as complete as spontaneously occurring egg laying (*e.g.* in *Aplysia*, bag cell extracts induced the laying of egg masses as large or larger than spontaneously laid egg masses; Pinsker and Dudek, 1977). In contrast, *Busycon* requires several injections before eggs are laid, and the egg laying episodes end abruptly when injections are discontinued, as opposed to the several days long egg-laying episodes that occur spontaneously (Magalhaes, 1948).

The differences between *Busycon* and the other gastropods may reflect different patterns of neurosecretion. In *Aplysia* (Kupfermann and Kandel, 1970) and *Lymnaea* (Vlieger *et al.*, 1980) hormone is secreted from neurons during synchronized electrical activity of several minutes duration, after which the neurosecretory cells

are quite inexcitable. Hormone is thus released in a comparatively short "bolus", comparable to that produced by an injection, and, therefore, the egg laying produced by neurosecretion and injection is comparable. In *Busycon*, the substance(s) causing egg laying apparently must be secreted over the entire episode, a situation differing from the short duration of action produced by injection of an extract.

This hypothesis raises interesting questions concerning the mechanism by which such secretion might be maintained over several days. Also, in view of the fact that the minimum effective dose to cause capsule laying was at least $^1/_{16}$ CNS, mechanisms that increase the synthesis of the substance causing egg laying may be necessary in order to have enough material to secrete over the entire episode. Alternatively, there may be more than enough material present for the entire egg laying episode, and the high dose (*i.e.* greater than $^1/_{16}$ CNS) needed in injection experiments may result from inactivation of the active agent during preparative procedures, lower efficiency of injection in the foot compared to the natural pattern and location of secretion, and/or lower sensitivity of the animal to the substance causing capsule laying than during spontaneous laying.

Previous experiments (Ram, 1977) showed that the *Busycon* capsule-laying substance is protease-sensitive and, therefore, likely to be a peptide, a result also demonstrated for the egg-laying hormone of *Aplysia* (Toevs & Brackenbury, 1969). Gel filtration indicates a similar molecular weight of the *Busycon* capsule laying substance to *Aplysia* egg-laying hormone (4385 daltons, Chiu *et al.*, 1979); however, the substances are not identical, since cross-injection experiments failed to induce egg or capsule laying. Although one might attribute the failure to obtain laying in cross-injection experiments in one direction to insufficient dosage, failure in both directions must mean the hormones are not identical. This conclusion can be drawn because similar extracts of each caused laying in con-specifics. The amount of *Aplysia* extract used (0.5 abdominal ganglion per recipient) caused laying in three out of three *Aplysia* in the present experiments and is two to five times greater than the threshold dose of 0.1 to 0.25 abdominal ganglion observed by Toevs (1970). The amount of *Busycon* extract used (one CNS per recipient) caused laying of egg capsules when injected into *Busycon* (Ram, 1977) and is four to sixteen times the threshold dose of $^1/_{16}$ to ¼ CNS observed in this paper. One could not have both more than enough of the hormone to obtain laying with conspecific injections into one species and insufficient amount for cross-injection into the other and simultaneously have more than enough of the *identical* hormone in the extracts of the other species and yet not enough when injected into the first species. This result is similar to observations on cross-injection experiments between *Aplysia* and *Pleurobranchaea* (Ram *et al.*, 1977) since the *Pleurobranchaea* egg-laying hormone is also similar in gel filtration behavior, yet it, too, fails to induce laying in *Aplysia*, and *vice versa*, in cross-injection experiments.

In contrast, *Strombus* CNS caused capsule laying in *Busycon*. In previous studies on interspecific induction of egg-laying behavior, cross-injection was successful between species in the same genus (Toevs, 1970) or in the same order (Ram, 1982b), but not between species in two different orders of Opisthobranchia (Ram, *et al.*, 1977; Ram, 1982b). Similarly, galactogenin (Goudsmit and Ram, 1982) is active between different species of *Helix* but not between species in the two orders of Pulmonata (Goudsmit, E. M., Oakland University, personal communication). Both *Strombus* and *Busycon* are in the subclass Prosobranchia; however, *Busycon* is in the order Neogastropoda, whereas *Strombus* is in the order Mesogastropoda. Thus, induction of capsule laying in *Busycon* by *Strombus* CNS is the first demonstration in gastropods of inter-order induction of a reproductive response controlled by a neuronal substance. This result may imply either a closer evolutionary

relationship between neogastropods and mesogastropods than has been demonstrated between other gastropod orders, or that there are atypical constraints on the evolution of the substance causing egg-capsule laying in prosobranchs. Purification, sequencing, and comparison of the substances involved, as has been done for *Aplysia* egg-laying hormone (Chiu *et al.*, 1979), would be a start in unraveling these molecular aspects of evolutionary relationships of gastropods.

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